

*Short Communication***Chip-based capillary electrophoresis profiling of olive pollen extracts used for allergy diagnosis and immunotherapy**

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**Abbreviations**

CRD: component resolved diagnosis

IHR: in-house reference extract

SIT: specific immunotherapy

SPT: skin prick test

**Keywords:** allergen, chip-based CE, extract, pollen, standardization, vaccine

**Word count:** 2730

Received: 03-Aug-2013; Revised: 16-May-2014; Accepted: 22-May-2014

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/elps.201300366.

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## Abstract

Standardization of protein extracts for clinical purposes represents an important task in order to maintain adequate reactivity, presence of the relevant allergens and safety among other factors. The main objective of this work was to explore the potential use of a chip-based automated capillary electrophoresis system commercially available to analyze several of the most common forms of allergenic extracts from olive pollen used in allergy clinics. These include experimental extracts prepared from olive pollens, in-house reference extracts, extracts designed for skin prick test assays, and a panel of vaccine variants aimed to specific immunotherapy. As a major conclusion of the study, chip-based capillary electrophoresis allowed in all cases to determine accurate protein profiles with different degrees of sensitivity, where several allergens (particularly the major olive pollen allergen Ole e 1) were easily recognized. Moreover, several purified allergens were also analyzed by this method, and proposed as specific standards for different purposes. In the present condition, the method can only provide the protein profile of the extracts respect to a pre-established standard extract, but not allergen identification. However, these and other future developments and applications are discussed.

Olive pollen represents a major source of allergy in the Mediterranean and several other emerging countries, affecting a relevant percentage of their population. Up to date, twelve allergens have been isolated and biochemically characterized from olive pollen, with a broad panel of molecular weights, isoelectric points, polysaccharide-associated profiles and biochemical characteristics [1]. Allergograms (patient's sensitization profiles revealed by Western blotting) are however, much more complex. Ole e 1, with three main variants of c.a.

18, 20, and 22 kDa, as well as other aggregated forms, has been described as the major olive pollen allergen and a marker of *Oleaceae*-associated allergy. This allergen, as well as the remaining olive pollen allergens where studied, displays a relevant molecular polymorphism. Furthermore, such allergen polymorphism is a common feature for many other species, and has been demonstrated to be closely associated (both in qualitative and quantitative terms) to the genetic origin and diversity of the original plant material used to prepare pollen extracts [2, 3]. This large heterogeneity of the allergenic sources involves a extremely careful labor of standardization of the olive (and other) extracts used for clinical purposes, mainly those used for SPT (skin prick testing) and the preparation of personalized vaccines for SIT (Specific ImmunoTherapy) [4]. Standardization is a critical procedure ensuring the efficacy and safety of the diagnosis and treatments, and this is one of the reasons why extremely efficient methods are currently under development, these including high sensitivity quantitation methods like Western blotting and ELISA (Enzyme-Linked ImmunoSorbent Assay) among other. A recently emerged concept, named CRD (Component-Resolved Diagnosis) is becoming a standard for clinical purposes. Methods based on CRD rely in the accurate use of pure allergen molecules either produced by recombinant expression or purification from natural allergen sources [5, 6]. Recently, we have developed a multiplex method for the simultaneous detection of multiple allergens from olive pollen extracts, which also allows identifying patient's reactivity in a simultaneous detection method [7, 8]. The present work aims to explore the advantages of capillary electrophoresis (CE) for the analysis of both experimental and commercial pollen protein extracts for allergy diagnosis, in the context of a commercially available automated electrophoresis station, which uses a chip format.

Material source for the study consist of olive (*Olea europaea* L.) mature pollen grains, collected from dehiscent anthers at the end of the flowering period by vigorously shaking the flowering shoots inside paper bags. Sampling was carried out from trees from different

cultivars ('Picual', 'Loaime', 'Lechín de Granada', 'Lucio', 'Manzanilla', 'Hojiblanca', 'Arbequina', 'Gordal', 'Frantoio') belonging to the olive germplasm bank of the Centro de Investigación y Formación Agraria (CIFA) 'Venta del Llano' (Mengíbar, Jaén, Spain). Total protein extract was isolated according to [8] with minor modification. Pollen samples (0.1 g) were resuspended in 2.5 mL extraction buffer consisting of 40 mM Tris-HCl (pH 7.0), 2% (v/v) Triton X-100, 60 mM dithiothreitol, and 10 µl of a protease inhibitor cocktail (Sigma-Aldrich, USA). Proteins were allowed to elute for 2 h at 4°C under continuous stirring. The resulting supernatants were desalted and delipidated in a PD-10 column (GE Healthcare Bio-Sciences AB, Sweden). Pollen proteins were then precipitated at -20°C overnight in a solution of 20% (w/v) tricarboxylic acid prepared in acetone. The resulting pellet was resuspended in 40 mM Tris-HCl (pH 8.8). Total protein content was estimated using the 2D Quant Kit (Amersham Biosciences, USA) according to the manufacturer's instructions.

For sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), total proteins (25 µg per sample) were mixed with an equal volume of 2x SDS sample buffer [9], boiled for 3 min prior to gel loading and separated by SDS-PAGE on Criterion™TGX™ Precast Gels (Bio-Rad, USA) using Criterion™ Cell apparatus (Bio-Rad, USA). After electrophoresis, the resulting gels were stained with Coomassie Brilliant blue (CBB) according to standard procedures. Gel documentation was carried out in an ImageScanner III (GE Healthcare Bio-Sciences AB) using the LabScan 6.0 software (GE Healthcare Bio-Sciences AB).

SDS-CE procedure was carried out using a commercial, automated electrophoresis station (Experion™, Bio-Rad). Samples were prepared by using the Experion™ Pro260 analysis kit (Bio-Rad) according to the manufacturer's instruction. Briefly, protein samples (50 ng each), were prepared by mixing 2 µl Pro260 sample buffer containing 3.2% β-mercaptoethanol with 4 µl sample, heated for 10 min at 95°C and then diluted with 84 µl mQ water. Both, samples and the Experion™ Pro260 protein ladder were loaded onto Experion™ Pro260 microfluidic

chips and analyzed into the CE station, by illuminating with a 635 nm (excitation) laser and measuring fluorescence intensity at 685 nm (laser-induced fluorescence) by using a photodiode detector. Running time of each experiment was approximately 30 min. The Experion<sup>TM</sup> software (v. 3.0) was used for the visualization of the results in both graph and simulated gel views. To ensure reproducibility, all samples were run at least in triplicate.

Both standard SDS-PAGE and SDS-CE analysis of experimental olive pollen extracts are depicted in Figure 1.

### Proposed location for Figure 1

The standard and the virtual gels obtained reveal the presence of complex band profiles for all the cultivars analyzed (Fig. 1A and B). As previously reported [10], noticeable differences among cultivars can be observed, particularly as regard to the presence and intensity of bands of c.a. 18-20 kDa (Fig. 1A) for standard SDS-PAGE, in a clear correspondence with bands of c.a. 27-30 kDa (Fig. 1B) for chip-based CE. These bands (normally two, corresponding to a non-glycosylated and a glycosylated form of protein) have been widely reported as the major olive pollen allergen Ole e 1 by using highly discriminant methods including Western-blotting with well-characterized monoclonal antibodies [10], and peptide mapping procedures [11]. Bands named “system peak” in all virtual gels and electropherograms is a cluster of signal generated in the commercial system by fluorescent detergent micelles. Figures 1C and 1D display fluorescence density electropherograms resulting from chip-based CE, corresponding to two representative extracts for high- and low-Ole e 1 content, respectively. The peaks matching the Ole e 1 allergen, likely in its non-glycosylated and glycosylated forms are clearly distinguishable in both cases. Reproducibility of chip-based CE was very high, and both the virtual gels and the electropherograms obtained for a given sample in separate runs were practically identical (data not shown).

Correspondence between standard SDS-PAGE and SDS-CE in terms of apparent molecular weights, and between these techniques and mass spectrometry is not absolute, even though the commercial SDS-CE system used here includes a number of internal controls, like the use of optimized recombinant protein standards (two of them, with molecular masses of 10 and 260 kDa are used to build a proper alignment of samples, and are repeated in every sample by the Experion<sup>TM</sup> software). However, additional considerations may apply therefore explaining such slight divergences. For example, running conditions for SDS-PAGE and chip-based CE, although similar (denaturing and reducing) may be slightly different. These differences might be even higher in the case of glycoproteins like Ole e 1. In this regard, SDS-PAGE offers a closer approach to mass spectrometry data for Ole e 1 than chip-based CE. For this and other additional reasons (as explained later) the inclusion of further controls and internal standards is highly advisable, and can be easily incorporated into the system.

The application of chip-based CE methods to extracts with clinical interest for allergy diagnosis and treatment is illustrated in Figure 2.

Fig. 2A shows the virtual electrophoretic profile of an in-house reference (IHR) extract of olive pollen (lane 1), used for pharmaceuticals companies as a highly concentrated, high quality, stable extract for comparison and quality control of further developed products, in comparison with an extract from a similar source (lane 2). Fig. 2B shows the virtual profiles of three different commercial olive pollen extracts developed by pharmaceutical companies in order to perform skin prick tests (SPTs, widely used as a diagnostic tool for allergy). Finally, Fig. 2C shows the virtual profiles of four commercial extracts used as personalized vaccines.

## Proposed location for Figure 2

All the products analyzed present sharp differences which can be analyzed in detail and provide plenty of useful information for standardization. First, clear differences in the total amount of protein present in the samples can be observed, starting with a range of high content of proteins in our experimental extracts, and then decreasing amounts of proteins in the IHR extract, then the SPT samples, and finally the low-protein vaccines. This is a good agreement with the average contents usually present in these types of samples. Overall, all samples present a relatively important concentration of the Ole e 1 allergen, although other bands are also represented. Lane 1 in Figs. 2A, B and C corresponds to different preparations from the same commercial company, and these profiles are therefore practically identical, thus demonstrating the reliability of the method. As regard to the vaccines (panel C), sample 1 corresponds to a vaccine prepared for sub-lingual administration, whereas the remaining three samples are ready for sub-cutaneous administration, and hence present slightly lower protein concentration. Finally, sample 4 corresponds to a mixed vaccine, prepared for immunotherapy of pollen allergy by using olive and grasses pollen extracts in a 1:1 proportion. In this lane, it is remarkable the presence of relevant bands other than Ole e 1, at higher molecular weights.

The information obtained by chip-based CE systems like the one used here can be displayed in multiple forms, and therefore used in multiple ways. For example, a more detailed analysis of the protein profiles of the vaccines can be performed just by adjusting the sensitivity of the system, as displayed in the virtual gels and electropherograms represented in Figs. 2D, and E-H, respectively, without the need of additional experimental runs.

Figure 3 shows protein profiles corresponding to three purified allergens from different sources: Ole e 1 purified from olive pollen, a commercial human profilin (Ole e 2 allergen-heterologous) and Cu,Zn-superoxide dismutase (Ole e 5 allergen-heterologous, with multiple isoforms) used as standard (unpublished). The information obtained from these specific runs, can be used for multiple purposes like peak identification and quantitation within complex extracts, as recurrently mentioned in the present communication.

### Proposed location for Figure 3

The use of a dedicated automated electrophoresis station also offers additional possibilities not widely reported here. As the first option, accurate quantitation of total protein in samples, and of particular peaks (or allergens) within a complex profile, can be achieved. The system uses the upper marker (260 kDa) as an internal standard for relative quantitation, and automatically provides a table with protein quantification for each one of the identified peaks. This information could be made even more accurate throughout simultaneous reading of well-quantitated purified allergens, as shown here (Fig. 3), and further comparative analysis. The system peak is not considered in the quantitative concentration estimation carried out by the system.

The sensitivity of the method (around 2.5ng/μl), comparable with that of colloidal Coomassie blue staining of SDS-PAGE gels, together with the minimal volume of sample needed (typical load of 4 μl), and other advantages like the reliability, and the short time of processing make procedures of chip-based CE fitting extremely well with the needs for allergenic extracts standardization.

Future implementations may include the development of dedicated methods for the identification and quantitation of specific allergens, and the adaptation of affinity or



immuno-electrophoresis variants to chip-based CE, which could be also performed using non-reducing, semi-native or even native conditions.

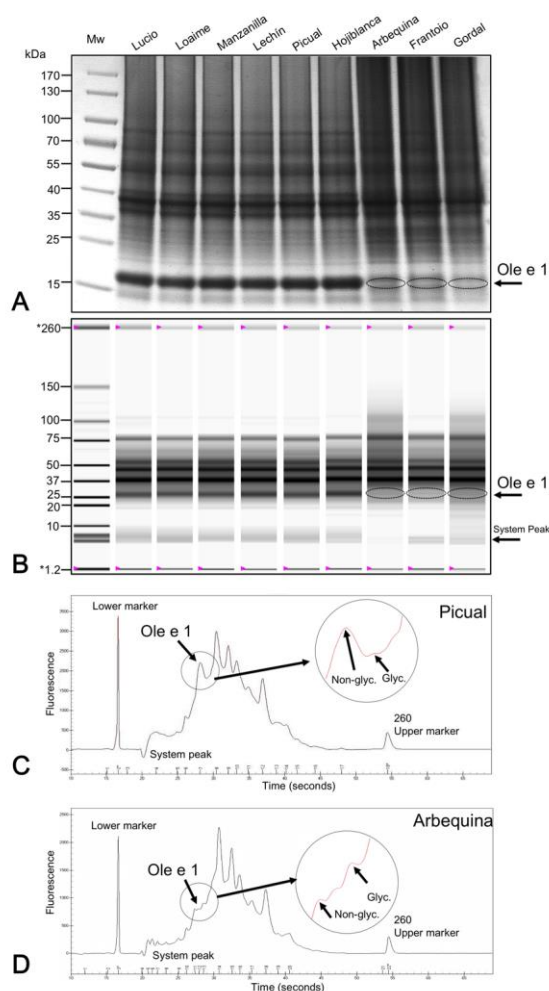
**Acknowledgements:** This work was supported by European Regional Development Fund (ERDF)-cofunded projects BFU2011-22779, P2010-AGR6274, P2010-CVI5767 and P2011-CVI-7487.

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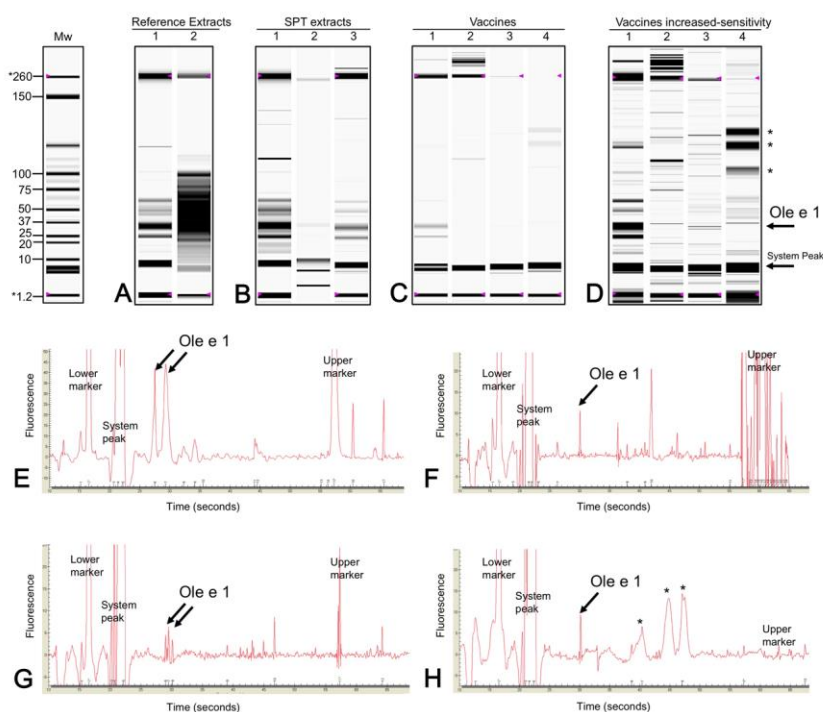
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**Figure 1.** Standard SDS-PAGE (A) and chip-based CE analysis (C-E) of experimental olive pollen extracts from different olive cultivars. Bands of c.a. 18-20 kDa (A) and c.a. 27-30 kDa (B) correspond to the major olive pollen allergen Ole e 1, with differential expression in those considered high-Ole e 1 content cultivars and those with low-Ole e 1 content (dotted ellipses). Internal controls for chip-based CE include lower and upper markers and a “system peak”. C and D: Electropherograms corresponding to a high-Ole e 1 and a low-Ole e 1, respectively, displaying differential Ole e 1 content. Non-glycosylated and glycosylated forms of Ole e 1 are clearly discriminated in selected zoom-view areas of the graphs.



**Figure 2.** Chip-based CE analysis (C-E) of most common types of extracts used for olive pollen diagnosis and therapy. Panel A shows the virtual electrophoretic profile of reference extracts, including an in-house reference (IHR) extract of olive pollen (lane 1), and a crude our experimental extract (lane 2). Panel B shows the virtual profiles of three different commercial olive pollen extracts for SPTs. Panel C shows the virtual profiles of four commercial extracts used as personalized vaccines. Panel D: as panel C, although displaying increased sensitivity after processing with the Experion<sup>TM</sup> software. E-H: Electropherograms corresponding to the increased-sensitivity analysis of lanes 1-4 from panel D, respectively. Lower and upper markers and the “system peak” are clearly labeled. Asterisks in lane 4 of Panel D mark additional bands present in this mixed extract (olive and grasses 1:1). Non-glycosylated and glycosylated forms of Ole e 1 are clearly discriminated and have similar intensity in E and G, whereas the non-glycosylated form seems to be more abundant in figures F and H.



**Figure 3.** Chip-based CE analysis (C-E) of three purified allergens from different sources. Panel A shows the virtual electrophoretic profile of Ole e 1 purified according to [12], whereas Panels B and C show those of Ole e 2-heterologous (recombinant human profilin, Abcam Ref. ab87760) and Ole e 5-heterologous (horseradish Cu,Zn-superoxide dismutase, Sigma catalogue no. S4636), respectively. Ole e 5 presents different isoforms clearly distinguishable. Panels D-F: electropherograms corresponding to the virtual gels for each purified allergen. Lower and upper markers and the “system peak” are clearly labeled.

